CHEMICAL CUES AFFECTING SUSCEPTIBILITY OF GORGONIAN CORALS TO FUNGAL INFECTION

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CHEMICAL CUES AFFECTING SUSCEPTIBILITY OF GORGONIAN CORALS TO FUNGAL INFECTION

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LIST OF ABBREVIATIONS

MIC	Minimum inhibitory concentration
FL10	Unidentified fungus strain isolated from seawater at Pickles Reef, Florida (N24 59.497 W80 24.551)
FL12	Another unidentified fungus strain also isolated from seawater at Pickles Reef, Florida (N24 59.497 W80 24.551)
BAHA2	Unidentified fungus strain isolated from seawater at Sweetings Cay, Bahamas (N26 33.65 W77 52.52)
NMR	Nuclear magnetic resonance
HMBC	Heteronuclear multiple bonding connectivity
HMQC	Heteronuclear multiple quantum correlation
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
δ	Chemical shift (in ppm) of a specific NMR peak

SUMMARY

Coral diseases have become more prevalent and destructive over the past 20 years, possibly due to an increase in stressful environmental factors that may weaken corals' defenses against disease. Aspergillosis is a disease caused by the fungus *Aspergillus sydowii*, which apparently infects only two species of gorgonian corals in the Caribbean Ocean (*Gorgonia ventalina* and *G. flabellum*). We hypothesized that the differential resistance to infection is caused by differences in chemical defenses among gorgonians and that *G. ventalina* and *G. flabellum* are less defended against fungal infection than other co-occurring gorgonians. In a survey of antifungal chemical defenses of 10 Caribbean gorgonians, freeze-dried gorgonian powders and extracts deterred fungal growth. The degree of growth inhibition varied among gorgonian species and among fungi. Extracts and powders generated from *G. ventalina* all strongly inhibited fungal growth. Since *G. ventalina* was predicted to have weak antifungal chemical defenses compared to gorgonians not known to suffer from aspergillosis, we concluded that gorgonian susceptibility to infection by *A. sydowii* is determined by factors other than, or in addition to, chemical defenses.

In order to investigate specific gorgonian antifungal strategies, we attempted to use bioassay-guided fractionation to isolate antifungal compounds from four gorgonians: *Gorgonia ventalina, Briareum asbestinum, Eunicea succinea,* and *Pseudopterogorgia americana*. We succeeded in isolating two antifungal compounds, diastereomers of 9,11-seco-24-hydroxydinosterol, from the gorgonian *P. americana*. This compound was previously identified by other groups, but this study is the first to establish its antifungal

activity. When one diastereomer of 9,11-seco-24-hydroxydinosterol was assayed against three different fungi, the growth of each was significantly inhibited. This suggests that at least this diastereomer may possess broad-spectrum antifungal activity.

CHAPTER 1
INTRODUCTION

In the last two decades, diseases have gained attention as prominent forces in shaping marine ecosystems (Harvell et al. 1999). Many marine systems still bear the scars of past disease outbreaks. For example, wasting disease nearly resulted in the extinction of eelgrass (*Zostera marina*) in the North Atlantic in the 1930s and 1980s (Muehlstein et al. 1991), and some eelgrass populations never recovered (Hemminga & Duarte 2000). The ensuing disappearance of eelgrass meadows caused waterfowl and fish populations to decline (Seymour et al. 2002). Caribbean coral reefs shifted from coral- to seaweed-dominated communities after the decimation of the sea urchin *Diadema antillarum* in the 1980s by a still-unknown pathogen (Lessios et al. 1984). White-band disease is one of many factors that have contributed to the demise of acroporid corals, which are major reef-builders in the Caribbean (Gladfelter 1982). Coral diseases are of particular scientific interest because the loss of these reef foundation species could lead to major community structure shifts on tropical reefs (Hughes 1994).

The majority opinion in a continuing debate appears to be that the number of coral diseases and the frequency of coral disease outbreaks are increasing (Harvell et al. 1999; Ward & Lafferty 2004). Certainly, ongoing damage caused by coral diseases—particularly in the Caribbean Sea—is widespread (reviewed by Harvell et al. 1999). Abiotic stressors such as increased water temperature can make corals more susceptible to disease (Harvell et al. 1999). In 1998, an El Niño event elevated water temperatures world-wide (Wilkinson et al. 1999), coinciding with severe coral bleaching in many places, including the Florida Keys (Harvell et al. 2001), Singapore, Australia, and the

Indian Ocean (Wilkinson et al. 1999; Harvell et al. 1999). Sedimentation may also increase stress on corals. In 1983 and 1987, the amount of dust blown across the Atlantic from Africa was unusually high (Prospero et al. 1996)—these two years also marked severe mortalities among several common coral genera in the Caribbean (Shinn et al. 2000). Overfishing promotes algal growth on reefs (Hughes 1994), and the resulting struggle for space and sunlight between corals and algae may stress corals and compromise their defenses against disease. Encroaching seaweeds may also serve as pathogen reservoirs. The bacterium that causes white plague disease in corals was found on numerous individuals of the seaweed *Halimeda opuntia* in Curaçao (Nugues et al. 2004). Nugues et al (2004) found that sections of *H. opuntia* transplanted onto the scleractinian coral *Montastrea faveolata* triggered white plague disease in this coral. Other sources of potential stress include pollutant input and the introduction of novel pathogens into a system (Harvell et al. 1999).

Elucidating the nature of coral diseases has proven to be difficult. Disease pathogens are often not readily cultured in the lab or they consist of microbial consortia that are not easily teased apart (Richardson 1998; Harvell et al. 1999). Pathogens have been identified for only 4 of 15 proposed coral diseases (Richardson et al. 2001). Aspergillosis is one such disease: it is caused by the soil fungus *Aspergillus sydowii* (Geiser et al. 1998), whose spores may be deposited in the Caribbean through agricultural runoff or Saharan dust (Shinn et al. 2000). Aspergillosis occurs throughout the Caribbean Sea (Kim & Harvell 2002), predominantly affecting the gorgonian corals *Gorgonia ventalina* and *G. flabellum* (Smith et al. 1996). Aspergillosis lesions on *Gorgonia* colonies are surrounded by dark purple tissue (Smith et al. 1996)—this

"purpling" symptom, caused by melanization and changes in sclerite concentration, is presumably induced by aspergillosis (Mullen et al. 2004). Aspergillosis can lead to partial or total death of the colony (Geiser et al. 1998).

There are over 40 species of gorgonian corals in the Caribbean (Bayer 1961), but only *Gorgonia ventalina* and *G. flabellum* have been documented as being visibly morbid from *Aspergillus sydowii* infection (Smith et al. 1996). This raises an important question: why are other gorgonians apparently unaffected by this disease? We proposed five competing hypotheses: (1) *G. ventalina* and *G. flabellum* lack specific antifungal defenses whereas other gorgonians are chemically defended against *Aspergillus sydowii*; (2) *Gorgonia* spp. do possess antifungal chemical defenses against *A. sydowii*, but these defenses are inadequate; (3) *A. sydowii* may be adapted for growth only on *Gorgonia* spp.; (4) other gorgonians may become infected with *A. sydowii*, but these outbreaks have not been observed or recorded; and (5) *Gorgonia* spp. may come in contact with more fungal spores because of their flatter, wider shape relative to other gorgonians. The first two hypotheses are tested in the current study.

Many marine invertebrates are rich in secondary metabolites (reviewed by Pawlik 1993). Some of these natural products act as chemical defenses to protect sessile, soft-bodied adult invertebrates or their larval offspring against predators (e.g. Pawlik et al. 1987; Lindquist 1996). Examples of chemically-defended marine invertebrates include marine worms (e.g. Kicklighter et al. 2003), tunicates (e.g. Joullié et al. 2003), nudibranchs (e.g. Avila et al. 2000), sponges (e.g. Kubanek et al. 2002), and cnidarians (e.g. O'Neal & Pawlik 2002). The use of chemical defenses as alternatives and supplements to structural defenses may optimize energy and resource allocation (Cronin

2001). With densities ranging from 5.4-25.1 colonies m^{-2} in the Caribbean (Goldberg 1973; Preston & Preston 1975), gorgonian corals do not appear to suffer heavy predation. Their main predators are gorgonian specialists: the mollusk *Cyphoma gibbosum*, the butterflyfish *Chaetodon capistratus,* the nudibranch *Tritonia hamnerorum,* and the bristleworm *Hermodice carunculata* all tend to inflict small-scale, localized damage on gorgonian colonies (Lasker 1985; Harvell & Suchanek 1987; Cronin et al. 1995; O'Neal & Pawlik 2002). Low predation rates on these animals may indicate that gorgonians are utilizing chemical and/or structural defenses. Pawlik et al. (1987) found that 19 of 37 lipid-soluble extracts of common Caribbean gorgonians were highly unpalatable to a generalist carnivore, the blue head wrasse (*Thalassoma bifasciatum*). Numerous feeding deterrent compounds have been identified from various gorgonians, and these metabolites are typically isoprenoid compounds (Coll 1992). Among the many structural variations of deterrent gorgonian terpenoid compounds, modifications can include halogenation, such as is common in erythrolides from the encrusting gorgonian *Erythropodium caribaeorum* (Fenical & Pawlik 1991), or complex ring structures such as cembranolide compounds from *Eunicea succinea* and pseudopterolide from *Pseudopterogorgia acerosa* (reviewed by Rodriguez 1995).

Secondary metabolites may also function to protect sessile marine invertebrates and plants from potential parasites—e.g. colonization or infection by bacteria, fungi, or algae (Targett et al. 1983; Wilsanand et al. 2001). Living gorgonians have few epibionts, but dead colonies or damaged branches are quickly covered by algae and other fouling organisms (Burkholder 1973; Gerhart et al. 1988). Extracts of numerous gorgonian species prevent fouling by diatoms (Targett et al. 1983; Wilsanand et al. 2001).

Some gorgonians also appear to use secondary metabolites to defend themselves against pathogenic microbes. In a study of the antimicrobial activity of extracts from 39 species of Caribbean gorgonians, 15 % of extracts suppressed the growth of three known invertebrate pathogens (all bacteria) and also suppressed multiple strains of bacteria isolated from dead or decaying gorgonians (Jensen et al. 1996). When extracts from 20 common Caribbean gorgonian species were assayed against several *Aspergillus* strains, the minimum inhibitory concentrations (MIC's) of extracts ranged from potent (MIC less than the natural concentration of extract) to non-potent (MIC greater than the natural concentration of extract) (Kim et al. 2000b). *Gorgonia ventalina* and *G. flabellum* were expected to have high MIC's since these gorgonians are known to be susceptible to aspergillosis; however, the MIC's of these two species fell in the middle of the range of gorgonians (Kim et al. 2000b). This suggests that resistance to aspergillosis may not be purely mediated by chemical cues, although the extraction methodology employed by Kim et al. (2000b) resulted in only highly lipophilic compounds being tested. We further investigated the role of gorgonian secondary metabolites in fungal infection by comparing antifungal activities of multiple species' extracts (at natural concentrations and encompassing a broad solubility range) against several fungi.

We also examined the specific antifungal chemical defense strategies of four species of gorgonians including *Gorgonia ventalina*, which showed strong antifungal activity in the survey of gorgonian extracts. We were successful in this endeavor for one gorgonian, *Pseudopterogorgia americana*. Knowing the identities of gorgonian antifungal metabolites may open the door for further investigation of topics such as defense induction and deterrence mechanisms of individual compounds.

CHAPTER 2
MATERIALS AND METHODS

2.1 Sample collection and extraction

Gorgonian samples were collected at the following locations: Pickles Reef, FL, USA (N24 59.497 W80 24.551); Conch Reef, FL, USA (N24 56.821 W80 27.341); Sweetings Cay, Bahamas (N26 33.65 W77 52.52); Little San Salvador, Bahamas (N24 32.68 W75 55.73); San Salvador, Bahamas (N24 01.14 W74 32.68); Plana Cays, Bahamas (N22 35.00 W73 36.00). A voucher specimen of each species was preserved in formalin and stored at Georgia Institute of Technology. The remaining samples were frozen immediately after collection and maintained at -20 ^{o}C until freeze-drying and extraction. Before this, a small portion of each species was thawed, rinsed, towel-dried, weighed, and its volume determined by volumetric displacement in water.

In order to minimize the degradation of biologically active compounds, all gorgonian samples were freeze-dried before extraction. Twenty-five milliliters of each species (based upon wet volume) were set aside after freeze drying, and these samples were ground to a powder with a Thomas-Wiley grinding mill (Thomas Scientific, New Jersey). The remaining samples were cut into pieces approximately 10 cm long and extracted in the following sequence of solvent systems: 1:1 water:methanol, 100% methanol, 1:1 methanol:dichloromethane, 2:1 dichloromethane:methanol, and the extracts were combined to yield a crude extract for each species.

2.2 Collection of Fungi

Seawater was collected from locations in the Bahamas and Florida and filtered through a 0.2 μm glass fiber filter (Millipore, Massachusetts). This filter was dried overnight in a Petri dish and then cut into approximately 1 cm^2 pieces. The filter pieces were plated on YPM medium (4 g/L mannitol, 2 g/L yeast extract, 2 g/L peptone, 16 g/L agar, 250 mg/L penicillin G, and 250 mg/mL streptomycin sulfate in 32 psu artificial seawater (Instant Ocean, Aquarium Systems, Ohio)) and incubated at 25 ^oC. Growing colonies were then transferred to fresh YPM plates, and several transfers were sometimes necessary to achieve a pure colony. Three of these fungal isolates were chosen for our experiments: designated strains FL10 (collected at Pickles Reef, Florida), FL12 (collected at Pickles Reef, Florida), and BAHA2 (collected at Sweetings Cay, Bahamas). Strains FL12 and BAHA2 are maintained at Georgia Institute of Technology at 25 ^oC on YPM medium.

2.3 Antifungal Assays

Crude extract from a 4 ml volume of each gorgonian species (hereafter called ml equivalent) was dissolved in 200 μl acetone. Four milliliters of molten YPM growth medium were added to each extract, stirred rapidly until the acetone evaporated, and 1 ml dispensed into a well of a 24-well plate (n = 3 per extract). Negative controls were prepared in the same way (but with acetone only) and interspersed with treatments.

Freeze-dried gorgonian powders were tested for antifungal activity in a similar way but without carrier solvent. Freeze-dried gorgonian powder (4 ml equivalent) was added to 4 ml of molten YPM medium. The powder/agar mixture was stirred to evenly

distribute the powder throughout the medium, and 1 ml aliquots were added to wells within 24-well plates (total of three wells per species—one well per plate). Negative controls were prepared without the addition of gorgonian powder.

A suspension of live fungal hyphae was prepared by cutting a small square approximately 1 cm^2 from a stock fungal lawn grown on YPM agar and homogenizing it with 1 ml of sterile DI water in a disposable, sterile tissue grinder (Fisher Scientific, Pennsylvania). Fungi used in the assays included: strains FL10 and BAHA2, a strain of *Aspergillus sydowii* not known to be pathogenic to *Gorgonia* spp. (strain FK1, provided by Dr. Drew Harvell, Cornell University), and a strain of *A. sydowii* known to be pathogenic to *Gorgonia* spp. (strain SS7, also provided by Dr. Drew Harvell). Each well was inoculated by making a small hole with a sterile pipette tip and then injecting 2 μL of the fungal suspension into the hole. The plates were incubated at 25 ^{o}C for 3-4 d or until growth on the positive controls reached at or near the edge of the well.

Percent cover of each well was determined from digital photographs taken with an Olympus C-3040 Zoom camera mounted to a dissecting scope (Olympus SZ-PT, Olympus, New York). Photographs were processed using ImageJ, a Java-based imaging software (National Institutes of Health), and the area of fungal growth was calculated by the Area Calculator plug-in (Rasband 2000). Percent cover was calculated by dividing the area of fungal growth by the total possible growth area for a well and multiplying by 100.

Due to small sample sizes, the percentages could not be transformed using the arcsine transformation (Zar 1999). Several of our assays involved multiple collections of the same species; therefore, we used nested analysis of variance (Zar 1999) to account for

variances among subsamples and among collections. Tukey-Kramer's multiple comparison post-hoc test (Tukey 1949) was used to calculate significance levels between species as well as between collections of the same species. In order to compare results from multiple bioassays, the mean percent fungal growth data points were each divided by the mean percent fungal cover of their corresponding control. This yielded values in terms of the proportion of control fungal cover. We also calculated the Spearman rank correlation between our fungal growth cover data (from Floridian gorgonian extracts tested against pathogenic *Aspergillus sydowii*) and the MIC's reported by Kim et al. Kim et al. 2000b for the species we used in our bioassays.

2.4 Bioassay-guided Fractionation of *Pseudopterogorgia americana* extract

We chose species for bioassay-guided fractionation by eliminating species not deterrent to fungal growth in the extract survey (*Pterogorgia citrina* and *P. guadalupensis*) and species for which the amount of crude extract was not sufficient for fractionation (*Plexaurella nutans* and *Plexaura homomalla*). Extracts from *Gorgonia ventalina* gradually lost activity as they were fractionated, so this species was eliminated as well. Antifungal compounds in the remaining three species, *Briareum asbestinum, Eunicea succinea*, and *Pseudopterogorgia americana*, were then pursued. The antifungal activities of *B. asbestinum* and *E. succinea* were spread out through multiple, separated fractions—the extracts from these two species were therefore saved for further separation at a later date. The extract from *P. americana* had consistently strong antifungal activity. Therefore, we proceeded with isolating antifungal compounds from this species.

Crude extract from *Pseudopterogorgia americana* was separated by liquid-liquid partitioning between petroleum ether and methanol/water (9:1), after which the aqueous fraction was sequentially partitioned between chloroform and methanol/water (3:2), between ethyl acetate and water, and between *n*-butanol and water (according to a modification of Kupchan et al. (1975)). Antifungal activity against the pathogenic strain of *Aspergillus sydowii* was confined to the chloroform-soluble fraction, so this fraction was subjected to reversed-phase column chromatography (Sep-Pak C18 column, Waters Corporation, Massachusetts) using a gradient of methanol/water (3:1) to 100% ethyl acetate. The antifungal fraction that eluted with 3:1 methanol:water was then subjected to repeated rounds of reversed-phase HPLC (Waters 1525 gradient pump; Waters 2996 photodiode array; Empower Chromatography Software, version 2; all manufactured by Waters Corporation, Massachusetts) with a Zorbax C-18 column (Agilent Technologies, California) until two pure antifungal compounds were isolated.

The structures of these antifungal compounds were elucidated by ^{13}C and ^{1}H NMR spectroscopy as well as HMBC, HMQC, DEPT, and COSY 2-D NMR methods (Bruker DRX500, Bruker, Massachusetts). These compounds showed the following NMR spectral characteristics: ^{1}H NMR (500MHz, C_6D_6), δ 0.63 (s, H_3-18), 0.77 (m, H-5), 0.85 (d, H_3-27), 0.87 (d, H_3-30), 0.90 (d, H_3-26), 0.92 (s, H_3-19), 0.98 (m, H-7), 1.01 (d, H_3-21), 1.10 (m, H_2-16), 1.15 (s, H_3-28), 1.21 (m, H-4), 1.23 (m, H-15), 1.24 (m, H-1), 1.34 (m, H-6), 1.45 (m, H-16), 1.51 (m, H-12), 1.54 (m, H-15), 1.55 (s, H_3-29), 1.58 (m, H-2), 1.60 (m, H-1), 1.68 (m, H-6), 1.73 (m, H-12), 1.74 (m, H-2), 1.74 (d, H-7), 1.75 (m, H_2-17), 2.47 (m, H-20), 2.67 (m, H-8), 2.70 (m, H-3), 2.77 (m, H-14), 3.71 (m, H-11), 3.82 (m, H-11), 5.50 (d, H-22); ^{13}C NMR (125 MHz, C_6D_6): δ 13.2 (C-29), 15.3 (C-

30), 16.6 (C-19), 17.1 (C-26), 17.2 (C-27), 17.9 (C-18), 21.3 (C-21), 23.9 (C-15), 24.5 (C-16), 24.6 (C-28), 25.3 (C-6), 31.1 (C-1), 31.4 (C-2), 32.6 (C-7), 33.5 (C-20), 34.3 (C-25), 39.0 (C-4), 41.1 (C-12), 42.4 (C-14), 44.0 (C-8), 45.6 (C-13), 48.9 (C-10), 50.7 (C-17), 51.7 (C-5), 59.2 (C-11), 75.3 (C-3), 77.0 (C-24), 128.0 (C-22), 137.3 (C-23), 216.2 (C-9). Molecular weights and molecular formulae were determined by mass spectrometry methods: electron impact mass spectrometry was carried out on a VG 70SE mass spectrometer (VG Instruments, acquired by Waters Corporation, Massachusetts) and electrospray ionization was performed with a QSTAR XL mass spectrometer (Applied Biosystems, California). SciFinder Scholar (version 2004.2, Chemical Abstracts Service, Ohio) was used to search the scientific literature for structural similarities between the isolated natural product and previously reported compounds.

CHAPTER 3
RESULTS

3.1 Survey of the Antifungal Activity of Caribbean Gorgonians

All Floridian and Bahamian freeze-dried gorgonian powders significantly deterred the growth of each fungus relative to controls (Figure 1). The fungal strains FL10 and BAHA2 appeared to be especially sensitive, with growth of these fungi reduced by 83-100 % relative to controls, when exposed to freeze-dried powders (Figures 1-2). Effects on *Aspergillus sydowii* differed among gorgonian species, some of which were collected during different months. The powders from different collections of a given species generally showed consistent potency; however, the potencies of powders from different collections of *Eunicea succinea* differed significantly. When Floridian and Bahamian powders were assayed against pathogenic *A. sydowii*, the potencies of *Gorgonia ventalina* powders were in the middle of the observed range of gorgonian powder potencies (Figure 1). Powders from Bahamian specimens of *G. ventalina* were also moderately deterrent towards non-pathogenic *A. sydowii* (Figure 1). In all other bioassays, *G. ventalina* freeze-dried powders were among the most deterrent powders tested.

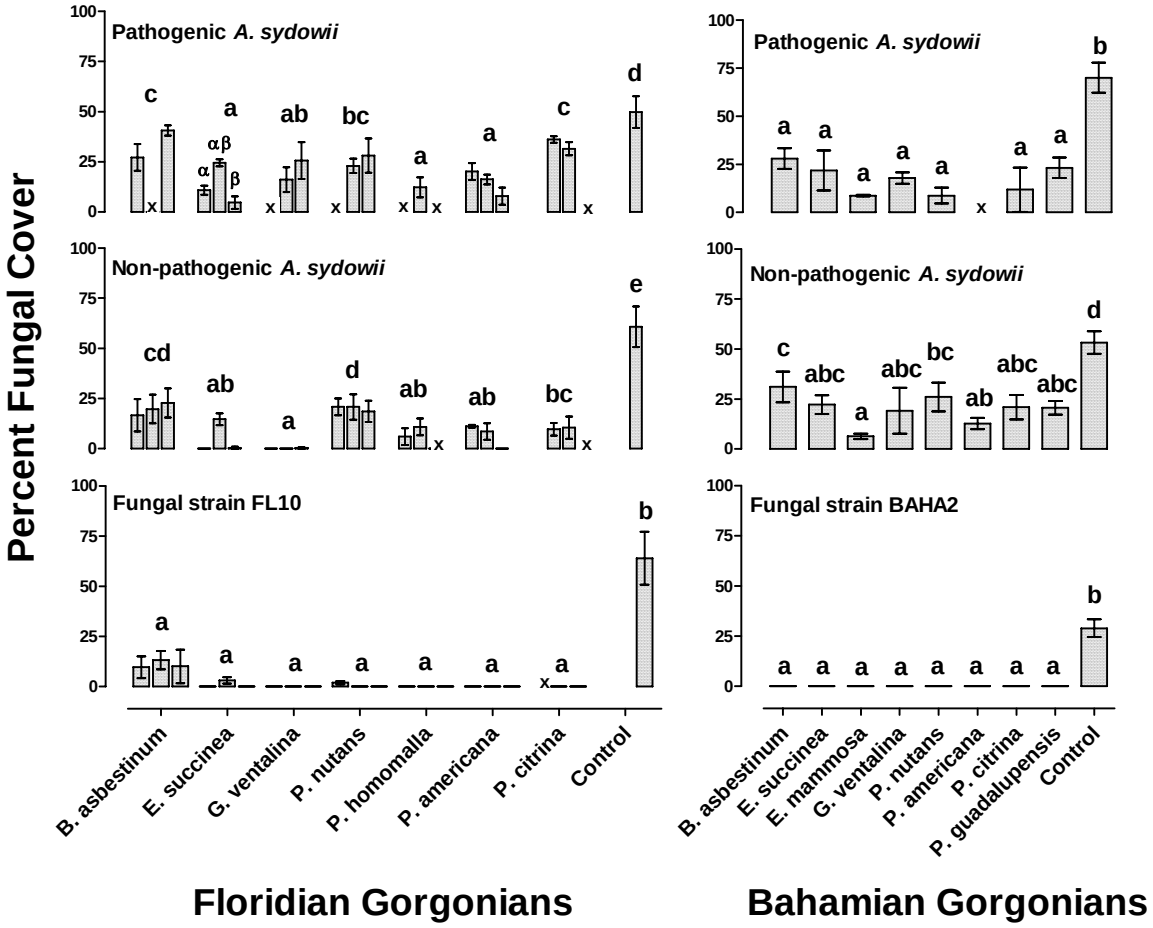

Figure 1. Effects of freeze-dried gorgonian powders on fungal growth Each bar represents one collection of a species (n = 3 sub-samples), and groups of bars indicate all of the collections for a species (error bars represent standard deviation of each collection). Shared letters indicate no significant difference at the species level ($p < 0.05$, nested ANOVA with Tukey-Kramer's post-hoc test). Greek letters indicate significant differences between collections of the same species ($p < 0.05$, nested ANOVA with Tukey-Kramer's post-hoc test). Greek letters are not shown if collections did not differ. x's indicate no data available for that species or collection.

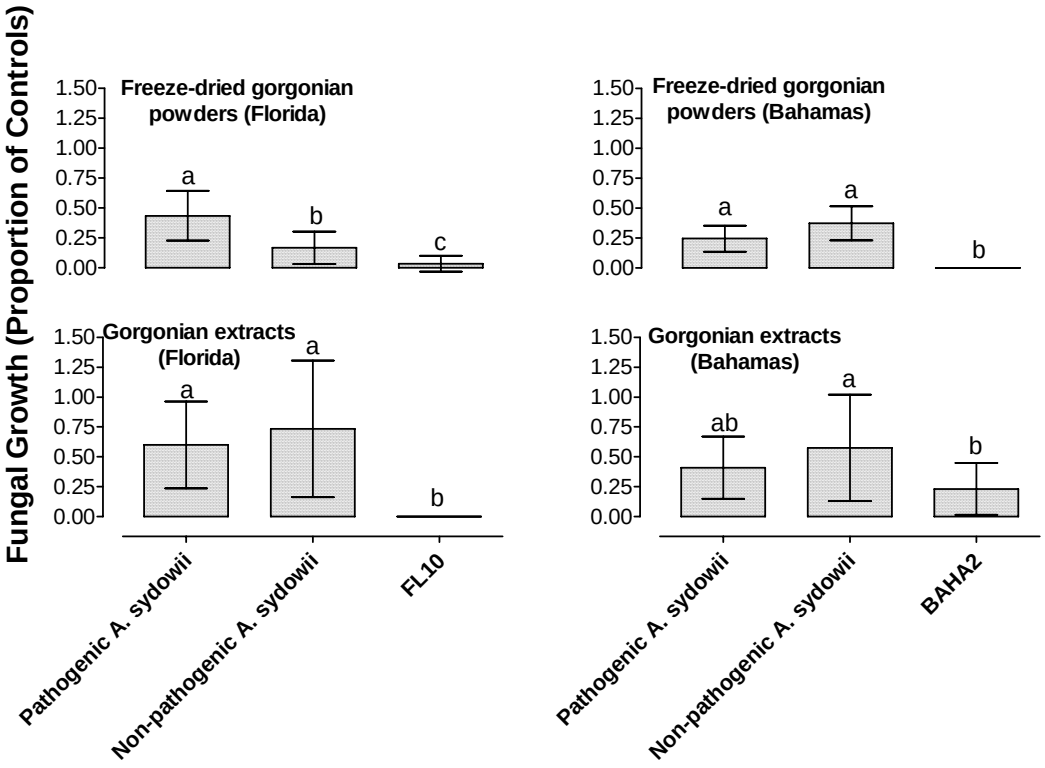

Figure 2. Overall effects of Floridian and Bahamian gorgonian powders and extracts on fungal growth. Shared letters indicate no significant difference between resistances of different fungi ($p < 0.05$). Error bars indicate standard deviation from mean. Data were analyzed with one-way ANOVA followed by Tukey-Kramer's post-hoc test.

The majority of gorgonian extracts tested were not significantly deterrent to all three fungi tested, but all extracts were deterrent against the strains FL10 and BAHA2 (Figure 3). Strain FL10 was more sensitive to gorgonian extracts than either strain of *Aspergillus sydowii* (paralleling the sensitivity of this strain to gorgonian powders). Strain BAHA2, on the other hand, was more sensitive to extracts than non-pathogenic *A. sydowii* but not more sensitive than pathogenic *A. sydowii* (Figure 2). Extracts from Floridian collections of *Gorgonia ventalina* were strongly deterrent to growth of all three fungi (Figure 3). Two out of the three collections of Bahamian *G. ventalina* extracts showed strong antifungal activity to all three fungi, but the third was conspicuously less deterrent against the non-pathogenic strain of *A. sydowii*. *Pterogorgia citrina* and *P. guadalupensis* extracts were noticeably ineffective at deterring growth of *A. sydowii*—these extracts were only deterrent to the FL10 and BAHA2 fungal strains (Figure 3). Three out of seven extracts from Floridian gorgonians (*G. ventalina, Briareum asbestinum*, and *Eunicea succinea*) were deterrent to a non-pathogenic strain of *Aspergillus sydowii*, and these same three gorgonians also inhibited growth of a pathogenic strain of *A. sydowii* (Figure 3). Four Bahamian extracts were also deterrent to non-pathogenic *A. sydowii* (*G. ventalina, E. mammosa, Plexaurella nutans,* and *Pseudopterogorgia americana*), and an additional three species significantly deterred the growth of pathogenic *A. sydowii* (*B. asbestinum, E. succinea,* and *Plexaura homomalla*) (Figure 3).

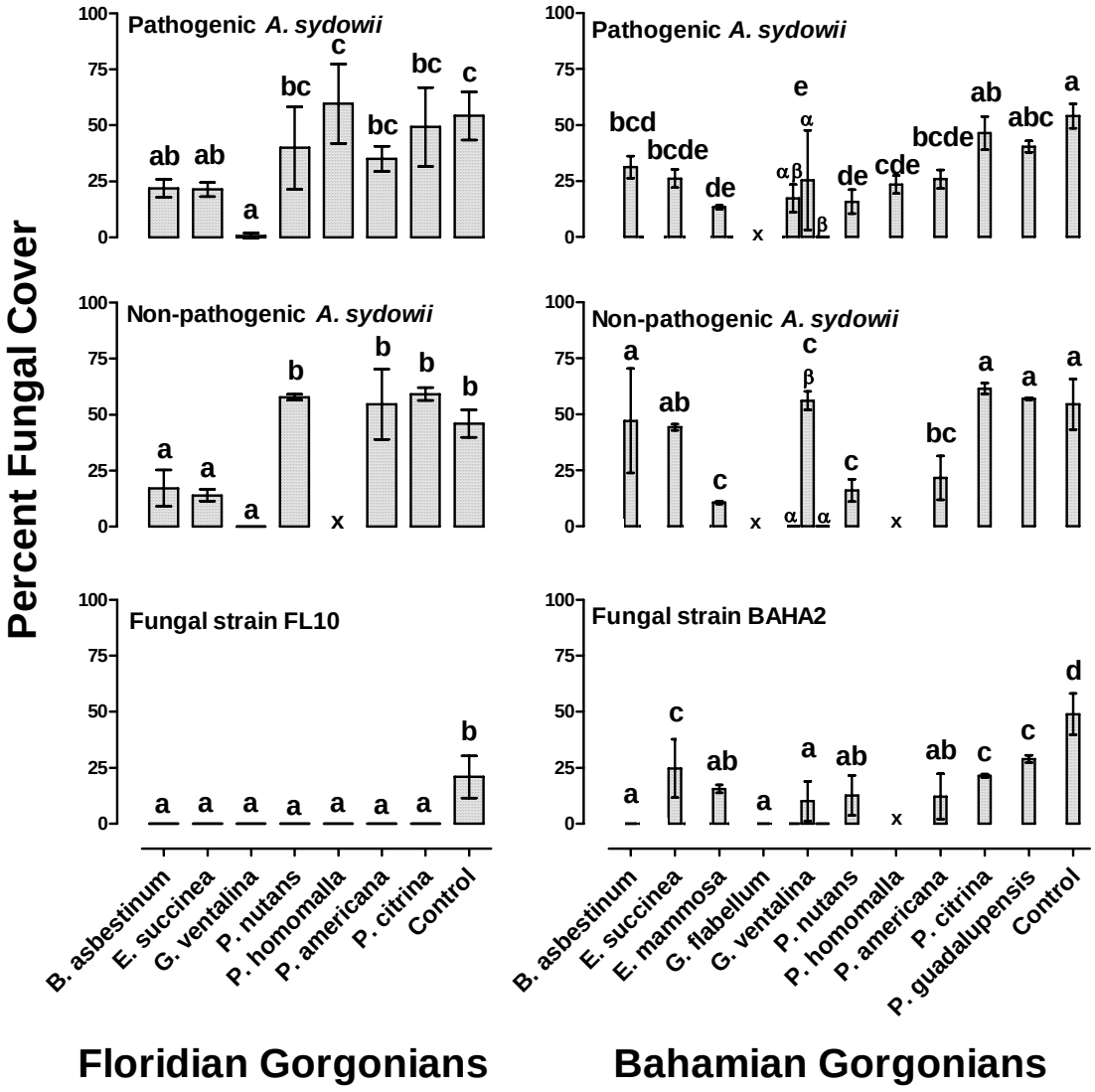

Figure 3. Effects of gorgonian extracts on fungal growth. *Gorgonia ventalina* was the only species for which multiple collections were extracted (indicated by a group of three bars, each representing one collection, n = 3 sub-samples; error bars represent standard deviation from mean of respective collection). Shared letters indicate no significant difference at the species level ($p < 0.05$, nested ANOVA with Tukey-Kramer's post-hoc test). Greek letters indicate significant differences between collections of the same species ($p < 0.05$, nested ANOVA with Tukey-Kramer's post-hoc test). Greek letters are not shown if collections did not differ. x's indicate no data available for that species.

There was a slight, non-significant positive correlation between the proportions of control fungal growth of our samples and the MIC's found by Kim et al. (2000b) (Figure 4).

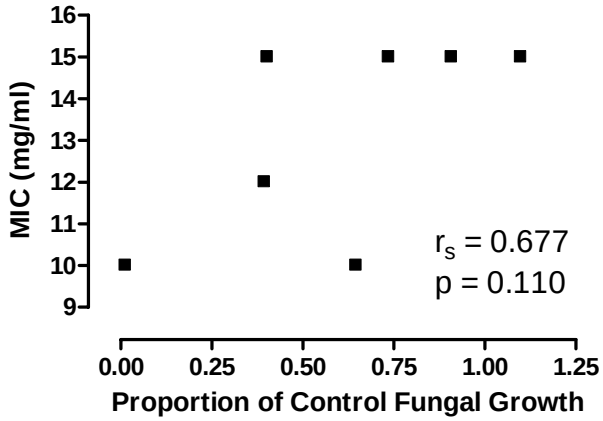

Figure 4. Comparison of gorgonian extract antifungal activity for this study (x-axis) versus Kim et al. (2000b) (y-axis) by Spearman rank correlation. Each data point represents one Floridian species common to both studies.

3.2 Bioassay-guided Fractionation of *Pseudopterogorgia americana* Extract

When crude extract of *Pseudopterogorgia americana* was partitioned into five fractions of differing polarity, only the chloroform-soluble fraction showed antifungal activity against pathogenic *Aspergillus sydowii* (Figure 5). Further separation of this antifungal fraction by reversed-phase flash column chromatography and repeated reversed-phase HPLC, coordinated with antifungal assays after each round of purification, eventually resulted in the isolation of two antifungal compounds (designated peaks A and B of the final HPLC purification; Figure 5). Purified peak B was significantly deterrent to the growth of pathogenic *A. sydowii* relative to controls (two-tailed t-test, $p = 0.007$; Figure 5) and to fungal strain BAHA2 (percent cover on agar with

peak B: 34.8 ± 2.2 %; control percent cover for BAHA2: 51.8 ± 6.7 %; two-tailed t-test, p = 0.014) and FL12 (percent cover on agar with peak B: 4.6 ± 1.9 %; control percent cover for FL12: 87.2 ± 22.1 %; two-tailed t-test with Welch's correction for unequal variances, p = 0.023). Peak A was not tested for antifungal activity against fungi other than *A. sydowii*, but demonstrated antifungal activity against pathogenic *A. sydowii* (two-tailed t-test, p = 0.002; Figure 5).

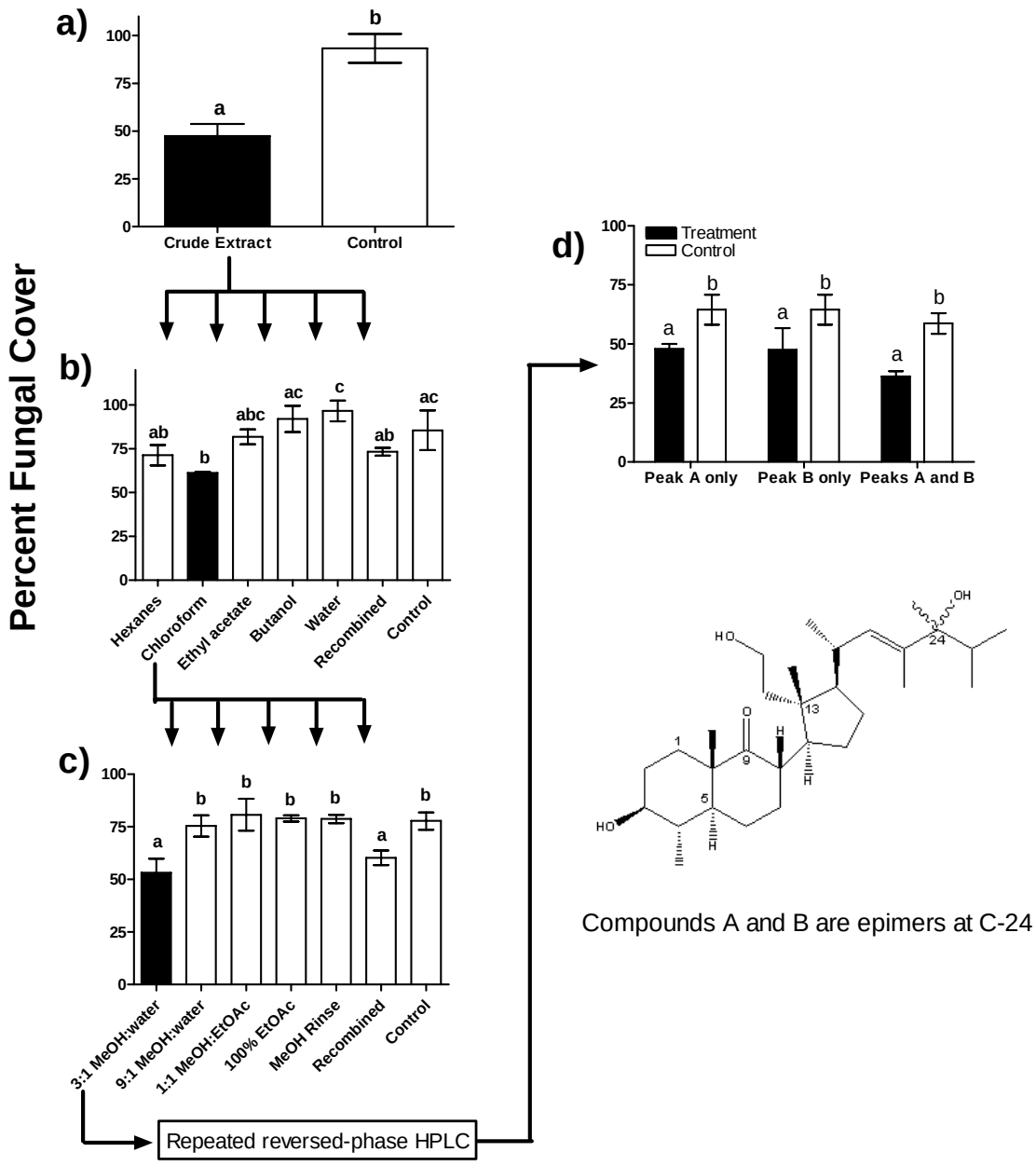

Figure 5. Fractionation of *Pseudopterogorgia americana* extract guided by antifungal assays with the pathogenic strain of *Aspergillus sydowii*. Shared letters indicate no significant difference (one-way ANOVA followed by Tukey-Kramer's post-hoc test). Other HPLC peaks were not active in antifungal assays (data not shown).

Both isolated antifungal compounds represented by HPLC peaks A and B yielded identical NMR and mass spectral data, but their slightly different yet consistent HPLC retention times (following multiple rounds of HPLC) indicated that they were not the same compound. The compound in HPLC peak A was isolated with a yield of 0.019 mg per ml of fresh gorgonian tissue (5.1×10^{-3} % of gorgonian dry mass), and the compound in HPLC peak B was isolated with a yield of 0.023 mg per ml of fresh gorgonian tissue (6.2×10^{-3} % of gorgonian dry mass). Combining the two compounds produced a marginal but non-significant increase in antifungal activity against pathogenic *Aspergillus sydowii* ($p = 0.072$, one-way ANOVA followed by Tukey-Kramer's multiple comparison test against peak B only; $p = 0.20$ for control of peaks A and B combined versus control of peaks A and B solo).

Mass spectrometric analysis of the compounds in HPLC peaks A and B using electrospray ionization in positive ion mode indicated that both compounds possessed molecular masses of 476 Da, from strong ion signals at 459.4 Da (representing protonation and loss of water) and 499.5 Da (representing a sodium adduct). Similarly, positive electron impact mass spectral analyses of the compounds in HPLC peaks A and B presented ion peaks at 458.3 Da (representing loss of water). By high-resolution electrospray ionization, the sodiated ions measured 499.3761 Da and 499.3758 Da for peaks A and B, respectively, which deviated only 0.4 and 1.0 ppm from the expected mass for $C_{30}H_{52}O_4Na$ (499.3763 Da). The ^{13}C NMR spectral data of both compounds indicated 30 carbons, including one ketone group, one carbon-carbon double bond, and three carbons attached to oxygen (see Materials and Methods for chemical shifts). Comparison of ^{1}H and ^{13}C NMR spectral data (Figures 6 and 7) with published data (He

et al. 1995; Miller et al. 1995), as well as analysis of 2D NMR spectral data, confirmed that our antifungal compounds were isomers of 9,11-seco-24-hydroxydinosterol (Figure 5).

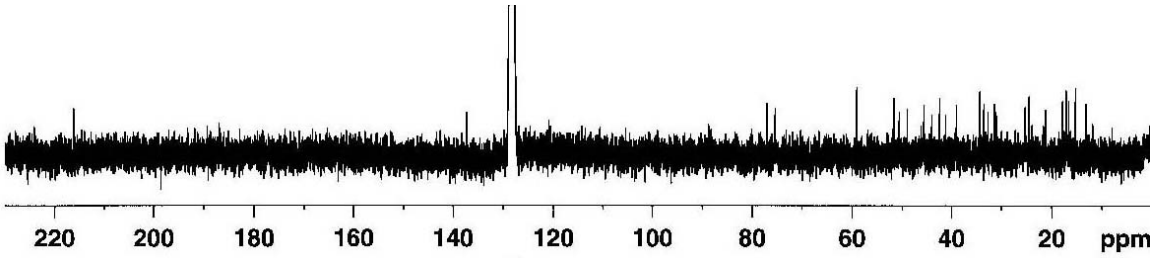

Figure 6. Carbon-13 NMR spectrum for peak B (125 MHz, C_6D_6) (spectral data were identical for peak A).

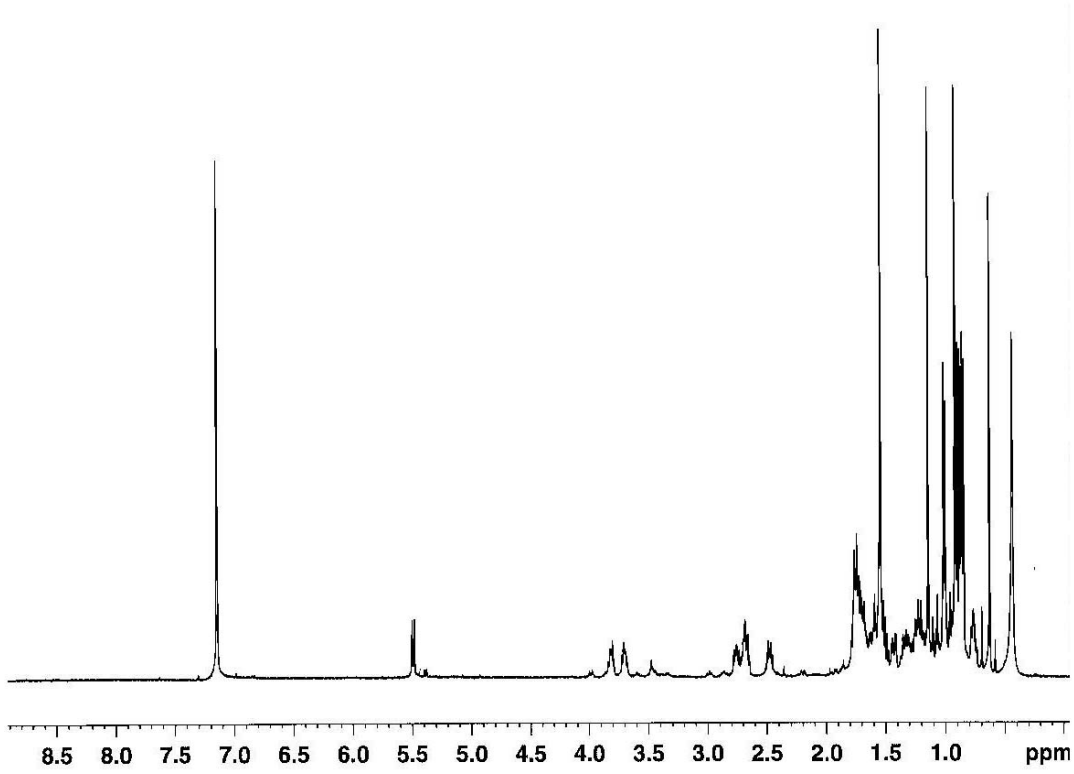

Figure 7. Proton NMR spectrum of peak B (500 MHz, C_6D_6) (spectral data were identical for peak A).

We predict that our two isolated compounds differed by being epimeric at the tertiary alcohol carbon on the side chain (C-24). Epimerization would result in the observed slightly different HPLC elution times of these two isomers and would be likely to occur very readily, due to the stability of a tertiary carbocation. Although diastereomers typically possess different chemical shifts for carbons and hydrogens near the epimeric center, the magnitude of chemical shift differences between diastereomers depends on the distance between the epimeric center and the nearest other chiral center. It seems plausible that C-24 epimers of 9,11-seco-24-hydroxydinosterol may possess chemical shifts so similar as to be indistinguishable, and given the identical mass spectral and NMR data, no other hypotheses seemed likely.

CHAPTER 4
DISCUSSION

Antifungal chemical defenses appear to be common among Caribbean gorgonians. When compared, our results were slightly positively correlated with the MIC's reported by Kim et al. (2000b); however, the correlation was not statistically significant ($p = 0.11$; Figure 4). This trend towards a positive correlation is not surprising: it would be expected that gorgonian species with low MIC's would be the same species that showed strong fungal growth inhibition at natural concentrations. In both studies, crude extracts from *Gorgonia ventalina* showed potent antifungal activity (Figure 3: fungal growth was reduced by 98.7 % against pathogenic *Aspergillus sydowii*; Kim et al. (2000b) reported an MIC < 10 mg/ml against pathogenic *A. sydowii*). Kim et al. (2000b) also found that *Pterogorgia citrina* and *Pterogorgia guadalupensis* had the highest MIC's in a range of gorgonian extracts tested against pathogenic *A. sydowii*—this is consistent with the lack of antifungal activity that we observed for these two species against both strains of *A. sydowii*. Since we cut our gorgonians into pieces before extraction such that inner-tissue compounds were extracted, our results are descriptive of gorgonian chemical defenses that inhibit the penetration of fungi through tissue, rather than defenses that inhibit fungal colonization of gorgonian surfaces. We concluded that antifungal chemical defenses are indeed prevalent in Caribbean gorgonians, but differences among potencies from extracts of gorgonians from different collections suggest that location, season, or other factors may influence these chemical defenses. Given the similarity between our findings and those of Kim et al. (2000b) and despite different extraction methodology, it appears that the more lipophilic-focused approach of

Kim et al. (Kim et al. 2000b) suffices to extract all of most antifungal chemical defenses from gorgonians. Although the current study is the first to characterize specific gorgonian compounds acting as antifungal defenses, other known gorgonian natural products are typically lipophilic (Blunt et al. 2005 and references therein).

Both the pathogenic and non-pathogenic strains of *Aspergillus sydowii* were considerably less sensitive to gorgonian extracts and freeze-dried powders than fungal strains FL10 and BAHA2. *A. sydowii* may have evolved resistance specifically to the antifungal defenses of gorgonians, or it may be broadly resistant to antifungal metabolites because of some unapparent metabolic characteristic. This may increase the impact of aspergillosis on Caribbean gorgonians if *A. sydowii* is better able to overcome host antifungal defenses, relative to other fungi.

If chemical defenses strongly influence host susceptibility to aspergillosis, *Gorgonia ventalina* extracts at natural concentrations would be expected to have little effect on growth of *Aspergillus sydowii*. However, we found that *G. ventalina* extracts were among the most antifungal of the extracts we tested (Figure 3). In fact, *G. ventalina* extracts from Florida almost completely suppressed growth of both strains of *A. sydowii* and strain FL10 (Figure 3). Again, our data are consistent with those of Kim et al. (2000b): in the latter study, extracts from only four species out of 20 had greater antifungal potency than *G. ventalina* when assayed against a pathogenic strain of *A. sydowii*. Therefore, susceptibility of *G. ventalina* to aspergillosis must be due to additional factors, not just the ability of gorgonian metabolites to inhibit fungal growth. From these data, we can rule out the first hypothesis outlined earlier in this paper: that

Gorgonia spp. lack fungal growth-inhibiting chemical defenses whereas other gorgonians possess such defenses.

The second hypothesis that we proposed—that *Gorgonia* spp. possess chemical defenses but these are inadequate to avoid infection—cannot be excluded. Since we found that extracts from several gorgonian species that do not suffer from aspergillosis possessed similar or weaker antifungal activity than *G. ventalina*, we propose that *G. ventalina* may possess other traits that increase its vulnerability to *A. sydowii* infections. It is also possible that other gorgonians possess additional defenses that *G. ventalina* lacks. For example, *Pseudopterogorgia americana* produces a slimy mucus on its surface (Bayer 1961) that may help this gorgonian slough off potential pathogens. We did not investigate other mechanisms by which secondary metabolites could deter fungi, such as the interruption of colonization or induction of aversive chemotactic responses in microbes (Jensen et al. 1996). It is possible that other gorgonians possess compounds that may inhibit fungi using these mechanisms. The relationship between chemical defenses and other traits that affect vulnerability to infection may be more complicated than originally anticipated.

The wide, flat shape of *Gorgonia* colonies is one of this genus's most striking morphological characteristics. Other gorgonian species are characterized by long individual branches as opposed to *Gorgonia*'s net-like, interconnected branches. Having such a large surface area perpendicular to the predominant current (Wainwright & Dillon 1969) could potentially increase pathogen exposure—*G. ventalina* and *G. flabellum* may be more vulnerable to infection because their colonies are more highly exposed than other gorgonians. The combination of orientation and flat surface area may also increase

the amount of damage that *Gorgonia* colonies receive during storm events, which could cause wounds that would be easily infected by fungi. Cronin (2001) proposed that an increase in abiotic stress may force seaweeds to allocate more resources to tissue maintenance and fewer towards chemical defense, and this might also occur in chemically defended invertebrates. However, antifungal activities of extracts from physically-damaged but uninfected gorgonians have not yet been investigated.

Gorgonian antifungal defenses could also be induced by pathogen infection (Kim et al. 2000a), analogous to induction of antimicrobial defenses in terrestrial plants (e.g. Kuc 1982, Järemo et al. 1999). Induction of resistance to herbivore grazing has been described in marine algae (Cronin & Hay 1996; Toth & Pavia 2000). Disease-induced chemical defenses in gorgonians are not well-documented, although Kim et al. (2000a) found very slight differences in *G. ventalina* crude extract potency at different positions around tissue lesions. Field inoculation experiments performed by Dube et al. (2002) showed that extracts from mature *G. ventalina* colonies that had been inoculated with *A. sydowii* were slightly more potently antifungal than extracts from control colonies. Dube et al. (2002) did observe that resistance among individual colonies varied widely—such variance could mask significant induction of chemical defenses.

We also noticed that gorgonian freeze-dried powders appeared to suppress fungal growth with greater potency than gorgonian extracts (Figures 1 and 3). We propose several hypotheses to explain the apparent differences between antifungal activities of gorgonian freeze-dried powders and extracts: (1) some deterrent compounds could have decomposed during the extraction process such that freeze-dried powders contained antifungal metabolites lost from extracts; (2) antifungal proteins present in freeze-dried

powders could have been denatured by our extraction solvents, resulting in lower potency of extracts; (3) highly polar antifungal compounds may not have been completely extracted from gorgonian tissue since the most polar extraction solvent system we used was 1:1 methanol:water, but these highly polar compounds would have been present in freeze-dried powders; and (4) the addition of freeze-dried powders to agar may have changed physical properties of the agar matrix (e.g. making the agar surface more dry and/or rough in texture), which could have negatively affected fungal growth relative to no-powder controls. This putative effect would be expected to have enhanced the apparent antifungal activity of powders relative to extracts (which may not have similarly affected the agar matrix in antifungal assays).

In the second part of our study, we pursued the identity of antifungal compounds of *Briareum asbestinum*, *Eunicea succinea, Gorgonia ventalina*, and *Pseudopterogorgia americana* using bioassay-guided fractionation with pathogenic *Aspergillus sydowii* as the test organism. We were successful at isolating an antifungal secosterol from one of these species, *Pseudopterogorgia americana,* which is a ubiquitous Caribbean gorgonian (Bayer 1961). We found that the source of this species's antifungal chemical defense was two diastereomers of 9,11-seco-24-hydroxydinosterol. Secondary metabolites produced by *P. americana* have been studied since the 1960's, and previously isolated compounds include sesquiterpenes as well as other secosterols (Weinheimer et al. 1968; Sica & Musumeci 2004). 9,11-Seco-24-hydroxydinosterol was first isolated from *P. americana* and described by He et al. (1995) and Miller et al. (1995), and our study is the first to demonstrate its antifungal properties. All but one stereocenter (C-24) of this compound were previously assigned by He et al. (1995) and Miller et al. (1995). We

found that two diastereomers (representing epimers at C-24—the previously unassigned stereocenter) each possessed antifungal activity, and combining the two resulted in a marginal, but non-significant increase in antifungal activity (Figure 5). These two compounds account for the antifungal potency of *P. americana* extracts, as no other chromatographic fractions possessed antifungal activity.

Other similar 9,11-secosteroids have been shown to be enzymatically derived from gorgosterol (Kerr et al. 1996). Gorgosterol, a marine sterol with a characteristic cyclopropane ring on its sidechain (Ciereszko et al. 1968), is produced by the dinoflagellate symbionts of gorgonians (Withers et al. 1982). In addition to providing nutrients to their hosts, these photosynthetic symbionts may help provide protection against fungal infection. Gorgonians may convert symbiont-produced gorgosterol into bioactive secosterols. 9,11-Seco-24-hydroxydinosterol has anti-inflammatory activity, and it also inhibits human protein kinase C (PKC) activity (Miller et al. 1995). PKC activity is important to fungi—including *Aspergillus* spp. (Simon et al. 1991; Morawetz et al. 1994; Morawetz et al. 1996)—so it is possible that inhibition of PKC is the mechanism by which 9,11-seco-24-hydroxydinosterol inhibits fungal proliferation. If PKC genes could be located in *Aspergillus sydowii*, it would be useful to test whether these genes are up- or down-regulated in the presence of 9,11-seco-24-hydroxydinosterol.

When we assayed one of the 9,11-seco-24-hydroxydinosterol epimers (HPLC peak B) against two fungal strains (FL12 and BAHA2), growth of each was significantly suppressed (two-tailed t-test; $p = 0.014$ for BAHA2; $p = 0.003$ for FL12). This same epimer also significantly deterred the growth of the pathogenic strain of *Aspergillus sydowii* (two-tailed t-test; $p = 0.007$, also see Figure 5). The reduction of fungal growth

by 9,11-seco-24-hydroxydinosterol varied among fungi, ranging from 27% to 95%. Given that all three tested fungi were significantly inhibited by 9,11-seco-24-hydroxydinosterol , it is likely that 9,11-seco-24-hydroxydinosterol possesses broad-spectrum antifungal activity. This compound may therefore protect *Pseudopterogorgia americana* from a variety of potentially pathogenic or saprophytic fungi.

Further research is necessary to determine what additional, non-chemical factors may influence the susceptibility of *Gorgonia ventalina* and *G. flabellum* to aspergillosis. Also, the identification of an enzymatically-produced antifungal compound provides more possibilities for investigating induction of antifungal chemical defenses. A more complete understanding of host-pathogen relationships is likely to assist the design of effective disease management procedures.

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